(51) International Patent Classification 6 : **C12N**	**A2**	(11) International Publication Number: **WO 97/23599**
		(43) International Publication Date: 3 July 1997 (03.07.97)

(21) International Application Number: PCT/US96/20094

(22) International Filing Date: 19 December 1996 (19.12.96)

(30) Priority Data:
60/009,119   22 December 1995 (22.12.95)   US
60/013,388   14 March 1996 (14.03.96)   US

(71) Applicants *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US). PURDUE RESEARCH FOUNDATION [US/US]; Purdue University, Office of Technology Transfer, 1650 Administration Building, Room 328, West Lafayette, IN 47907-1650 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: CHAPPLE, Clint [CA/US]; 2210 Robinhood Lane, West Lafayette, IN 47906 (US).

(74) Agents: FLOYD, Linda, A. et al.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

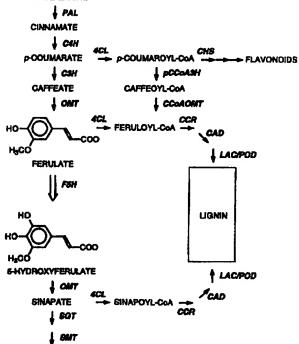(54) Title: A METHOD FOR REGULATION OF PLANT LIGNIN COMPOSITION

(57) Abstract

A method is disclosed for the regulation of lignin composition in plant tissue. Plants are transformed with a gene encoding an active F5H gene. The expression of the F5H gene results in increased levels of syringyl monomer providing a lignin composition more easily degraded with chemicals and enzymes.

PHENYLALANINE → PAL → CINNAMATE → C4H → p-COUMARATE → C3H → CAFFEATE → OMT → FERULATE → F5H → 5-HYDROXYFERULATE → OMT → SINAPATE → SGT → SMT → SINAPOYL MALATE

p-COUMARATE →4CL→ p-COUMAROYL-CoA →CHS→ FLAVONOIDS
p-COUMAROYL-CoA → pCCoASH → CAFFEOYL-CoA → CCoAOMT → FERULOYL-CoA (4CL) → CCR → CAD → LAC/POD → LIGNIN
SINAPATE →4CL→ SINAPOYL-CoA →CCR→ CAD → LAC/POD

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic	SD	Sudan
CF	Central African Republic		of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

TITLE
A METHOD FOR REGULATION OF PLANT LIGNIN COMPOSITION
FIELD OF INVENTION

The present method relates to the field of molecular biology and the

5   regulation of protein synthesis through the introduction of foreign genes into plant
genomes. More specifically, the method relates to the modification of plant lignin
composition in a plant cell by the introduction of a foreign plant gene encoding an
active ferulate-5-hydroxylase (F5H) enzyme. Plant transformants harboring the
F5H gene demonstrate increased levels of syringyl monomer residues in their

10   lignin, a trait that is thought to render the polymer more susceptible to
delignification.

BACKGROUND

Lignin is one of the major products of the general phenylpropanoid
pathway and is one of the most abundant organic molecules in the biosphere

15   (Crawford, (1981) Lignin Biodegradation and Transformation, New York: John
Wiley and Sons). In nature, lignification provides rigidity to wood and is in large
part responsible for the structural integrity of plant tracheary elements. Lignin is
well suited to these capacities because of its physical characteristics and its
resistance to biochemical degradation. Unfortunately, this same resistance to

20   degradation has a significant impact on the utilization of lignocellulosic plant
material (Whetten et al., *Forest Ecol. Management* 43, 301, (1991)).

The monomeric composition of lignin has significant effects on its chemical
degradation during industrial pulping (Chiang et al., *Tappi*, 71, 173, (1988). The
guaiacyl lignins (derived from ferulic acid) characteristic of softwoods such as pine,

25   require substantially more alkali and longer incubations during pulping in
comparison to the guaiacyl-syringyl lignins (derived from ferulic acid and sinapic
acid) found in hardwoods such as oak. The reasons for the differences between
these two lignin types has been explored by measuring the degradation of model
compounds such as guaiacylglycerol-β-guaiacyl ether, syringylglycerol-β-guaiacyl

30   ether, and syringylglycerol-β-(4-methylsyringyl) ether (Kondo et al.,
Holzforschung, 41, 83, (1987)) under conditions that mimic those used in the
pulping process. In these experiments, the mono- and especially di-syringyl
compounds were cleaved three to fifteen times faster than their corresponding
diguaiacyl homologues. These model studies are in agreement with studies

35   comparing the pulping of Douglas fir and sweetgum wood where the major
differences in the rate of pulping occurred above 150 °C where arylglycerol-β-aryl
ether linkages were cleaved (Chiang et al., *Holzforschung*, 44, 309, (1990)).

Another factor affecting chemical degradation of the two lignin forms may
be the condensation of lignin-derived guaiacyl and syringyl residues to form
diphenylmethane units. The presence of syringyl residues in hardwood lignins
leads to the formation of syringyl-containing diphenylmethane derivatives that

5   remain soluble during pulping, while the diphenylmethane units produced during
softwood pulping are alkali-insoluble and thus remain associated with the cellulosic
products (Chiang et al., *Holzforschung*, 44, 147, (1990); Chiang et al.,
Holzforschung, 44, 309, (1990)). Further, it is thought that the abundance of
5-5'-diaryl crosslinks that can occur between guaiacyl residues contributes to

10 resistance to chemical degradation. This linkage is resistant to alkali cleavage and
is much less common in lignin that is rich in syringyl residues because of the
presence of the 5-*O*-methyl group in syringyl residues. The incorporation of
syringyl residues results in what is known as "non-condensed lignin", a material
that is significantly easier to pulp than condensed lignin.

15   Similarly, lignin composition and content in grasses is a major factor in
determining the digestibility of lignocellulosic materials that are fed to livestock
(Jung, H.G. & Deetz, D.A. (1993) Cell wall lignification and degradability *in*
Forage Cell Wall Structure and Digestibility (H.G. Jung, D.R. Buxton, R.D.
Hatfield, and J. Ralph eds.), ASA/CSSA/SSSA Press, Madison, WI.). The

20 incorporation of the lignin polymer into the plant cell wall prevents microbial
enzymes from having access to the cell wall polysaccharides that make up the wall.
As a result, these polysaccharides cannot be degraded and much of the valuable
carbohydrates contained within animal feedstocks pass through the animals
undigested. Thus, an increase in the dry matter of grasses over the growing season

25 is counteracted by a decrease in digestibility caused principally by increased cell
wall lignification. From these examples, it is clear that the modification of lignin
monomer composition would be economically advantageous.

The problem to be overcome, therefore, is to develop a method for the
creation of plants with increased levels of syringyl residues in their lignin to

30 facilitate its chemical degradation. Modification of the enzyme pathway
responsible for the production of lignin monomers provides one possible route to
solving this problem.

The mechanism(s) by which plants control lignin monomer composition has
been the subject of much speculation. As mentioned earlier, gymnosperms do not

35 synthesize appreciable amounts of syringyl lignin. In angiosperms, syringyl lignin
deposition is developmentally regulated: primary xylem contains guaiacyl lignin,
while the lignin of secondary xylem and sclerenchyma is guaiacyl-syringyl lignin
(Venverloo, *Holzforschung* 25, 18 (1971); Chapple et al., *Plant Cell* 4, 1413,

(1992)). No plants have been found to contain purely syringyl lignin. It is still not clear how this specificity is controlled; however, at least five possible enzymatic control sites exist, namely caffeic acid/5-hydroxyferulic acid O-methyltransferase (OMT), F5H, (hydroxy)cinnamoyl-CoA ligase (4CL), (hydroxy)cinnamoyl-CoA

5   reductase (CCR), and (hydroxy)cinnamoyl alcohol dehydrogenase (CAD). For example, the substrate specificities of OMT (Shimada et al., *Phytochemistry*, 22, 2657, (1972); Shimada et al., *Phytochemistry*, 12, 2873, (1973); Gowri et al., *Plant Physiol.*, 97, 7, (1991); Bugos et al., *Plant Mol. Biol.* 17, 1203, (1992)) and CAD (Sarni et al., *Eur. J. Biochem.*, 139, 259, (1984); Goffner et al., *Planta.*, 188,

10   48, (1992); O'Malley et al., *Plant Physiol.*, 98, 1364, (1992)) are correlated with the differences in lignin monomer composition seen in gymnosperms and angiosperms, and the expression of 4CL isozymes (Grand et al., *Physiol. Veg.* 17, 433, (1979); Grand et al., *Planta.*, 158, 225, (1983)) has been suggested to be related to the tissue specificity of lignin monomer composition seen in

15   angiosperms.

        Although there are at least five possible enzyme targets that could be exploited, only OMT and CAD have been investigated in recent attempts to manipulate lignin monomer composition in transgenic plants (Dwivedi et al., *Plant Mol. Biol.* 26, 61, (1994); Halpin et al., *Plant J.* 6, 339, (1994); Ni et al.,

20   *Transgen. Res.* 3, 120 (1994); Atanassova et al., *Plant J.* 8, 465, (1995); Doorsselaere et al., *Plant J.* 8, 855, (1995)). Most of these studies have focused on sense and antisense suppression of OMT expression. This approach has met with variable results, probably owing to the degree of OMT suppression achieved in the various studies. The most dramatic effects were seen by using homologous

25   OMT constructs to suppress OMT expression in tobacco (Atanassova et al., *supra*) and poplar (Doorsselaere et al., *supra*). Both of these studies found that as a result of transgene expression, there was a decrease in the content of syringyl lignin and a concomitant appearance of 5-hydroxyguaiacyl residues. As a result of these studies, Doorsselaere et al., (WO 9305160) disclose a method for the

30   regulation of lignin biosynthesis through the genomic incorporation of an OMT gene in either the sense or anti-sense orientation. In contrast, Dixon et al. (WO 9423044) demonstrate the reduction of lignin content in plants transformed with an OMT gene, rather than a change in lignin monomer composition. Similar research has focused on the suppression of CAD expression. The conversion of

35   coniferaldehyde and sinapaldehyde to their corresponding alcohols in transgenic tobacco plants has been modified with the incorporation of an *A. cordata* CAD gene in anti-sense orientation (Hibino et al., *Biosci. Biotechnol. Biochem.*, 59, 929, (1995)). A similar effort aimed at antisense inhibition of CAD expression

generated a lignin with increased aldehyde content, but only a modest change in lignin monomer composition (Halpin et al., *supra*). This research has resulted in the disclosure of methods for the reduction of CAD activity using sense and anti-sense expression of a cloned CAD gene to effect inhibition of endogenous CAD

5   expression in tobacco [Boudet et al., (U.S. 5,451,514) and Walter et al., (WO 9324638); Bridges et al., (CA 2005597)]. None of these strategies increased the syringyl content of lignin, a trait that is correlated with improved digestibility and chemical degradability of lignocellulosic material (Chiang et al., *supra*; Chiang and Funaoka, *Holzforschung* 44, 309 (1990); Jung et al., *supra*).

10   Although F5H is also a key enzyme in the biosynthesis of syringyl lignin monomers it has not been exploited to date in efforts to engineer lignin quality. In fact, since the time of its discovery over 30 years ago (Higuchi et al., *Can. J. Biochem. Physiol.*, 41, 613, (1963)) there has been only one demonstration of the activity of F5H published (Grand, C., *FEBS Lett.* 169, 7, (1984)). Grand

15   demonstrated that F5H from poplar was a cytochrome P450-dependent monooxygenase (P450) as analyzed by the classical criteria of dependence on NADPH and light-reversible inhibition by carbon monoxide. Grand further demonstrated that F5H is associated with the endoplasmic reticulum of the cell. The lack of attention given to F5H in recent years may be attributed in general to

20   the difficulties associated with dealing with membrane-bound enzymes, and specifically to the lability of F5H when treated with the detergents necessary for solubilization (Grand, *supra*). The most recent discovery surrounding the F5H gene has been made by Chapple et al., *(supra)* who reported a mutant of *Arabidopsis thaliana* L. Heynh named *fah1* that is deficient in the accumulation of

25   sinapic acid-derived metabolites, including the guaiacyl-syringyl lignin typical of angiosperms. This locus, termed FAH1, encodes F5H. The cloning of the gene encoding F5H would provide the opportunity to test the hypothesis that F5H is a useful target for the engineering of lignin monomer composition.

In spite of sparse information about F5H in the published literature,

30   Applicant has been successful in the isolation, cloning, and sequencing of the F5H gene. Applicant has also demonstrated that the stable integration of the F5H gene into the plant genome, where the expression of the F5H gene is under the control of a promoter other than the gene's endogenous promoter, leads to an altered regulation of lignin biosynthesis.

35   SUMMARY OF THE INVENTION

The present invention provides isolated nucleic-acid fragments comprising the nucleotide sequences which correspond to SEQ ID NO.:1 and SEQ ID NO.:3 encoding an active plant F5H enzyme wherein the enzyme has the amino acid

sequence encoded by the mature functional protein which corresponds to SEQ ID NO.:2 and wherein the amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the function of the F5H enzyme.

The invention further provides a chimeric gene causing altered

5   guaiacyl:syringyl lignin monomer ratios in a transformed plant, the gene comprising a nucleic acid fragment encoding an active plant F5H enzyme operably linked in either sense or antisense orientation to suitable regulatory sequences. The nucleic acid fragments are those described above.

Also provided is a method of altering the activity of F5H in a plant by

10 means of transforming plant cells in a whole plant with a chimeric gene causing altered guaiacyl:syringyl lignin monomer ratios in a transformed plant cell, wherein the gene is expressed; growing said plants under conditions that permit seed development; and screening the plants derived from these transformed seeds for those that express an active F5H gene or fragment thereof.

15 A method is propvided of altering the activity of F5H enzyme in a plant by (i) transforming a cell, tissue or organ from a suitable host plant with the chimeric gene desribed above wherein the chimeric gene is expressed; (ii) selecting transformed cells, cell callus, somatic embryos, or seeds which contain the chimeric gene; (iii) regenerating whole plants from the transformed cells, cell callus, somatic

20 embryos, or seeds selected in step (ii); (iv) selecting whole plants regenerated in step (iii) which have a phenotype characterized by (1) an ability of the whole plant to accumulate compounds derived from sinapic acid or (2) an altered syringyl lignin monomer content relative to an untransformed host plant.

The invention additionally provides a method of altering the composition of

25 lignin in a plant by means of stably incorporating into the genome of the host plant by transformation a chimeric gene causing altered guaiacyl:syringyl lignin monomer ratios in a transformed plant; expressing the incorporated gene such that F5H is expressed and wherein guaiacyl:syringyl lignin monomer ratios are altered from those ratios of the untransformed host plant.

30 BRIEF DESCRIPTION OF THE FIGURES AN SEQUENCE LISTING

Figure 1 illustrates the biosynthesis of monomeric lignin precursors via the general phenylpropanoid pathway.

Figure 2 is an illustration of the pBIC20-F5H cosmid and the F5H overexpression construct (pGA482-35S-F5H) in which the F5H gene is expressed

35 under the control of the constitutive cauliflower mosaic virus 35S promoter.

Figure 3 shows an analysis of sinapic acid-derived secondary metabolites in wild type, the *fah1-2* mutant, and independently-derived transgenic *fah1-2* plants

5

carrying the T-DNA derived from the pBIC20-F5H cosmid, or the pGA482-35S-F5H overexpression construct.

Figure 4 shows the impact of F5H overexpression by comparing the steady state levels of F5H mRNA in wild type, the *fah1-2* mutant, and independently-
5   derived transgenic *fah1-2* plants carrying the T-DNA derived from the 35S-F5H overexpression construct.

Figure 5 shows a GC analysis of lignin nitrobenzene oxidation products to illustrate the impact of F5H overexpression on lignin monomer composition in the wild type, the *fah1-2* mutant, and a *fah1-2* mutant carrying the T-DNA derived
10 from the 35S-F5H overexpression construct.

Figure 6 illustrates a Southern blot analysis comparing hybridization of the F5H cDNA to *Eco*RI digested genomic DNA isolated from wild type *Arabidopsis thaliana* and a number of *fah1* mutants.

Figure 7 is a Northern blot analysis comparing hybridization of the F5H
15 cDNA to RNA isolated from wild type *Arabidopsis thaliana* and a number of *fah1* mutants.

Figure 8 shows the genomic nucleotide (SEQ ID NO.:3) and amino acid (SEQ ID NO.:2) sequences of the *Arabidopsis* F5H gene and the F5H enzyme that it encodes.

20   Applicant(s) have provided three sequence listings in conformity with 37 C.F.R. 1.821-1.825 and Appendices A and B ("Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences") and in conformity with "Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications" and Annexes I and II to the Decision of
25   the President of the EPO, published in Supplement No 2. to OJ EPO, 12/1992.

The sequence of the *Arabidopsis thaliana* F5H cDNA is given in SEQ ID NO.:1 and the sequence of the *Arabidopsis thaliana* F5H genomic clone is given in SEQ ID NO.:3. The sequence of the F5H protein is given in SEQ ID NO.:2.

<u>DETAILED DESCRIPTION OF THE INVENTION</u>

30   The present invention provides a gene that encodes F5H, a key enzyme in lignin biosynthesis. The invention further provides a method for altering the lignin composition in plants by transforming plants with the F5H gene wherein the gene is expressed and causes an increased conversion of ferulic acid to sinapic acid thereby increasing the syringyl content of the lignin polymer.

35   The effect in plants of lignin compositions containing higher syringyl monomer content is that the lignin is more susceptible to chemical delignification. This is of particular use in the paper and pulp industries where vast amounts of energy and time are consumed in the delignification process. Woody plants

transformed with an active F5H gene would offer a significant advantage in the delignification process over conventional paper feedstocks. Similarly, modification of the lignin composition in grasses by the insertion and expression of a heterologus F5H gene offers a unique method for increasing the digestibility of

5   livestock feed. Maximizing the digestibility of grasses in this manner offers great potential economic benefit to the farm and agricultural industries.

Plants to which the Invention may be Applied

The invention provides a gene and a chimeric gene construct useful for the transformation of plant tissue for the alteration of lignin monomer composition.

10 Plants suitable in the present invention comprise plants that naturally lack syringyl lignin or those that accumulate lignin with a high guaiacyl:syringyl ratio. Plants suitable in the present invention also comprise plants whose lignin could be modified using antisense transformation constructs that reduce the syringyl content of the transgenic plants' lignin if such an alteration were desirable.

15   Suitable plants may include but are not limited to alfalfa (*Medicago* sp.), rice (*Oryza* sp.), maize (*Zea mays*), oil seed rape (*Brassica* sp.), forage grasses, and also tree crops such as eucalyptus (*Eucalyptus* sp.), pine (*Pinus* sp.), spruce (*Picea* sp.) and poplar (*Populus* sp.), as well as *Arabidopsis* sp. and tobacco (*Nicotiana* sp.).

20 Definitions

As used herein the following terms may be used for interpretation of the claims and specification.

The term "FAH1" refers to the locus or chromosomal location at which the F5H gene is encoded. The term "*FAH1*" refers to the wild type allele of the gene

25 encoding the F5H gene. The *term "fah1"* refers to any mutant version of that gene that leads to an altered level of enzyme activity, syringyl lignin content or sinapate ester content that can be measured by thin layer chromatography, high performance liquid chromatography, or by *in vivo* fluorescence.

"Gene" refers to a nucleic acid fragment that expresses a specific protein,

30 including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to the gene as found in nature with its own regulatory sequences.

A "chimeric gene" refers to a gene comprising heterogeneous regulatory and coding sequences.

35   An "endogenous gene" refers to the native gene normally found in its natural location in the genome.

A "foreign gene" or "transgene" refers to a gene not normally found in the host organism but one that is introduced by gene transfer.

The term "promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition site for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are

5   involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other.

10   As used herein, suitable "regulatory sequences" refer to nucleotide sequences located upstream (5'), within, and/or downstream (3') of a coding sequence, which control the transcription and/or expression of the coding sequences in conjunction with the protein biosynthetic apparatus of the cell. These regulatory sequences include promoters, translation leader sequences, transcription

15   termination sequences, and polyadenylation sequences.

The term "T-DNA" refers to the DNA that is transferred into the plant genome from a T-DNA plasmid carried by a strain of *Agrobacterium tumefaciens* that is used to infect plants for the purposes of plant transformation.

The term "T-DNA plasmid" refers to a plasmid carried by *Agrobacteriurn*

20   *tumefaciens* that carries an origin of replication, selectable markers such as antibiotic resistance, and DNA sequences referred to as right and left borders that are required for plant transformation. The DNA sequence that is transferred during this process is that which is located between the right and left T-DNA border sequences present on a T-DNA plasmid. The DNA between these borders

25   can be manipulated in such a way that any desired sequence can be inserted into the plant genome.

The term "ferulate-5-hydroxylase" or "F5H" will refer to an enzyme in the plant phenylpropanoid biosynthetic pathway which catalyzes the conversion of ferulate to 5-hydroxyferulate and permits the production of sinapic acid and its

30   subsequent metabolites, including sinapoylmalate and syringyl lignin.

The terms "encoding" and "coding" refer to the process by which a gene, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce an active enzyme. It is understood that the process of

35   encoding a specific amino acid sequence includes DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is

therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are

5   also contemplated. For example, alterations in the gene sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic

10   residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal

15   portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity in the encoded products.

20   Moreover, the skilled artisan recognizes that sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (2X SSC, 0.1% SDS, 65 °C), with the sequences exemplified herein.

The term "expression", as used herein, refers to the production of the protein product encoded by a gene. "Overexpression" refers to the production of a

25   gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms.

"Transformation" refers to the transfer of a foreign gene into the genome of a host organism and its genetically stable inheritance. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation and particle-

30   accelerated or "gene gun" transformation technology as described in U.S. 5,204,253.

The term "plasmid rescue" will refer to a technique for circularizing restriction enzyme-digested plant genomic DNA that carries T-DNA fragments bearing a bacterial origin of replication and antibiotic resistance (encoded by the

35   β-lactamase gene of *E. coli*) such that this circularized fragment can be propagated as a plasmid in a bacterial host cell such as *E. coli*.

The term "lignin monomer composition" refers to the relative ratios of guaiacyl monomer and syringyl monomer found in lignified plant tissue.

9

The Phenylpropanoid Biosynthetic Pathway

The lignin biosynthetic pathway is well researched and the principal pathways are illustrated in Figure 1. Lignin biosynthesis is initiated by the conversion of phenylalanine into cinnamate through the action of phenylalanine

5   ammonia lyase (PAL). The second enzyme of the pathway is cinnamate-4-hydroxylase (C4H), a cytochrome P450-dependent monooxygenase (P450) which is responsible for the conversion of cinnamate to *p*-coumarate. The second hydroxylation of the pathway is catalyzed by a relatively ill-characterized enzyme, *p*-coumarate-3-hydroxylase (C3H), whose product is caffeic acid. Caffeic acid is

10   subsequently *O*-methylated by OMT to form ferulic acid, a direct precursor of lignin. The last hydroxylation reaction of the general phenylpropanoid pathway is catalyzed by F5H. The 5-hydroxyferulate produced by F5H is then *O*-methylated by OMT, the same enzyme that carries out the *O*-methylation of caffeic acid. This dual specificity of OMT has been confirmed by the cloning of the OMT gene, and

15   expression of the protein in *E. coli* (Bugos et al., (1991) *supra*; Gowri et al., (1991) *supra*).

The committed steps of lignin biosynthesis are catalyzed by 4CL, (hydroxy)cinnamoyl CoA reductase (CCR) and CAD, which ultimately generate coniferyl alcohol from ferulic acid and sinapoyl alcohol from sinapic acid.

20   Coniferyl alcohol and sinapoyl alcohol are polymerized by extracellular oxidases to yield guaiacyl lignin and syringyl lignin respectively, although syringyl lignin is more accurately described as a co-polymer of both monomers.

Although ferulic acid, sinapic acid, and in some cases *p*-coumaric acid are channeled into lignin biosynthesis, in some plants these compounds are precursors

25   for other secondary metabolites. In *Arabidopsis*, sinapic acid serves as a precursor for lignin biosynthesis but it is also channeled into the synthesis of soluble sinapic acid esters. In this pathway, sinapic acid is converted to sinapoylglucose which serves as an intermediate in the biosynthesis of sinapoylmalate (Figure 5). Sinapic acid and its esters are fluorescent and may be use as a marker of plants deficient in

30   those enzymes needed to produce sinapic acid (Chapple et al., *supra*).

Identification of the FAH1 Locus and *fah1* Alleles

A series of mutants of *Arabidopsis* that fail to accumulate sinapoylmalate have been identified and have been collectively termed *fah1* mutants. The fluorescent nature of sinapoylmalate permits the facile identification of sinapic acid

35   esters by thin layer chromatography (TLC) followed by observation under ultraviolet (UV) light). The fluorescence of sinapoylmalate can also be visualized *in vivo* because sinapoylmalate is accumulated in the adaxial leaf epidermis. Wild type *Arabidopsis* exhibits a pale blue fluorescence under UV while *fah1* mutants

appear dark red because of the lack of the blue fluorescence of sinapoylmalate and the fluorescence of chlorophyll in the subtending mesophyll (Chapple et al., *supra*).

A TLC-based mutant screen of 4,200 ethyl methanesulfonate-mutagenized *Arabidopsis* plants identified a number of independent mutant lines that

5 accumulated significantly lower levels of sinapoylmalate. The mutations in these lines were identified as *fah1-1* through *fah1-5*. The *in vivo* UV-fluorescence visual screen was used to identify more mutant lines carrying the *fah1* mutation. Two of these mutants (*fah1-6* and *fah1-7*) were selected from EMS-mutagenized populations. One mutant line (*fah1-8*) was selected from among a mutant

10 population generated by fast-neutron bombardment (Nilan, R. A. *Nucl. Sci. Abstr.*, 28(3), 5940 (1973); Kozer et al., *Genet. Pol.*, 26(3),367, (1985)). A final mutant line, (*fah1-9*) was identified using the same technique from a T-DNA tagged population of plants. Before further analysis, each mutant line was backcrossed at least twice to the wild type and homozygous lines were established.

15 To determine whether the newly isolated mutant lines were defective at the same locus, that is, within the gene encoding F5H, genetic complementation experiments were performed. In these tests, each mutant line was crossed *to fah1-2* which is known to be defective in F5H. In each case, the newly isolated mutant line was used as the female parent and was fertilized with pollen from a

20 *fah1-2* homozygous mutant. A reciprocal cross was also performed using *fah1-2* as the female parent, and the new mutant line as the pollen donor. The seeds from these crosses were collected several weeks later, and were planted for subsequent analysis. The progeny were analyzed for sinapoylmalate production by TLC, high pressure liquid chromatography and by observation under UV light. From these

25 crosses, all of the F1 progeny examined were sinapoylmalate-deficient, indicating that all of the mutations identified were allelic.

The *fah1-9* line was selected for further study because of the presence of the T-DNA insertion within the F5H gene. The T-DNA insertion within the FAH1 locus facilitated the cloning of the flanking Arabidopsis DNA which could then be

30 used to retrieve the wild type F5H gene from cDNA and genomic libraries (Meyer et al., *Proc. Natl. Acad. Sci. USA*, 93, 6869 (1996)).

Cloning of the FAH1 Locus

A fragment of DNA from the FAH1 locus was isolated from the T-DNA tagged *fah1-9* mutant using the technique of plasmid rescue (Meyer et al., *supra*).

35 The technique of plasmid rescue is common and well known in the art and may be used to isolate specific alleles from T-DNA transformed plants (Behringer, et al., *Plant Mol. Biol. Rep.*,10, 190,(1992)). Briefly, the vector used to generate the T-DNA tagged population of *Arabidopsis* carries sequences required for

autonomous replication of DNA in bacteria and sequences that confer antibiotic resistance. Once this DNA is integrated into the plant genome, specific restriction endonuclease digests can be employed to generate fragments that can be circularized, ligated, and transformed into *E. coli*. Circularized DNA from the

5   T-DNA will generate functional plasmids that confer antibiotic resistance to their bacterial hosts such that they can be identified by growth on selective media. Those plasmids that are generated from the sequences including the right and left borders will also carry with them the plant genomic sequences flanking the T-DNA insertion. Plasmids generated from either of the T-DNA borders that carry

10   flanking DNA sequences can be identified by analyzing the products of diagnostic restriction enzyme digests on agarose gels. The plasmids with flanking sequences can then serve as a starting point for cloning plant sequences that share homology to the DNA at the point of T-DNA insertion (Behringer, et al., *supra*).

Plasmid rescue was conducted using *Eco*RI-digested DNA prepared from

15   homozygous *fah1-9* plants. *Eco*RI-digested genomic DNA was ligated and then electroporated into competent DH5α *E. coli*. DNA from rescued plasmids was further digested with both *Eco*RI and *Sal*I and the digests were analyzed by gel electrophoresis to identify plasmids that contained flanking *Arabidopsis* DNA. A *Sac*II-*Eco*RI fragment from this rescued plasmid was used to identify an F5H clone

20   from an Arabidopsis cDNA library (Newman, T. et al., *Plant. Physiol.* 106, 1241, (1994)).

DNA Sequencing of the F5H cDNA and genomic clones

Sequence analysis of the F5H cDNA and genomic clones was performed on plasmid DNA manually using a United States Biochemical Sequenase Kit v. 2.0, on

25   a DuPont Genesis® 2000 sequencer or on an Applied Biosystems 373A DNA sequencer, using standard vector-based sequencing oligonucleotides or custom-synthesized oligonucleotides as appropriate. The sequence of the *Arabidopsis thaliana* F5H cDNA is given in SEQ ID NO.:1 and the sequence of the *Arabidopsis thaliana* F5H genomic clone is given in SEQ ID NO.:3.

30   The F5H cDNA contains a 1560 bp open reading frame that encodes a protein with a molecular weight of 58,728. The putative ATG initiation codon is flanked by an A at -3 and a G at +4, in keeping with the nucleotides commonly found flanking the initiator methionine in plant mRNAs (Lutcke et al., *EMBO J.* 6, 43, (1987)). Immediately following the inferred initiator methionine is a 17 amino

35   acid sequence containing nine hydroxy amino acids (Figure 8). The subsequent fifteen amino acid sequence is rich in hydrophobic amino acids; eleven hydrophobic · residues comprised of phenylalanine, isoleucine, leucine and valine residues. This hydrophobic stretch is immediately followed by an Arg-Arg-Arg-Arg putative stop

transfer sequence. F5H also shares significant sequence identity with other P450s. Most notable is the stretch between Pro-450 and Gly-460. This region contains eight residues that comprise the heme-binding domain and are highly conserved among most P450s, one exception being allene oxide synthase from *Linum*

5   *usitatissimum* (Song et al., *Proc. Natl. Acad. Sci. USA 90*, 8519, (1993)). The Pro-450 to Gly-460 region contains Cys-458 in F5H, which by analogy is most likely the heme binding ligand in this enzyme.

Transformation of *fah1-2 Arabidopsis* and Restoration of Sinapoylmalate Accumulation

10   The identity of the F5H gene was confirmed by complementation of the *fah1-2* mutant with a genomic clone and a construct where the F5H genomic coding sequence was expressed under the control of the cauliflower mosaic virus 35S promoter. Briefly, the F5H cDNA was used as a probe to screen a transformation competent library (Meyer et al., (1994) *Science*, 264, 1452-1455)

15   for genomic clones. Using this method, a cosmid clone (pBIC20-F5H) was isolated that carried a 17kb genomic insert containing the inferred start and stop codons of the F5H gene (Figure 2). The portion of this cosmid carrying the F5H open reading frame was excised from the cosmid and subcloned into a vector in which it was operably linked to the cauliflower mosaic virus 35S promoter

20   (pGA482-35S-F5H) (Figure 2). Both the original cosmid and this derivative plasmid construct were electroporated into *Agrobacterium tumefaciens* and were used to transform *fah1-2* mutants. Success of the transformations was evidenced by TLC assays demonstrating sinapoylmalate accumulation in leaf tissues of the *fah1-2* transformants carrying the T-DNA from the pBIC20-F5H cosmid or the

25   pGA482-35S-F5H plasmid (Figure 3). These data clearly indicated that the gene encoding F5H had been identified.

Modification of Lignin Composition in Plants Transformed With F5H Under the Control of the Cauliflower Mosaic Virus 35S Promoter

Arabidopsis plants homozygous for the *fah1-2* allele were transformed with

30   *Agrobacterium* carrying the pGA482-35S-F5H plasmid which contains the chimeric F5H gene under the control of the constitutive cauliflower mosaic virus 35S promoter (Odell, et al., *Nature* 313, 810-812, (1985)). Independent homozygous transformants carrying the F5H transgene at a single genetic locus were identified by selection on kanamycin-containing growth media, grown up in

35   soil and plant tissue was analyzed for lignin monomer composition. Nitrobenzene oxidation analysis of the lignin in wild type, *fah1-2*, and transformants carrying the T-DNA from the pGA482-35S-F5H construct revealed that F5H overexpression as measured by northern blot analysis (Figure 4) led to a significant increase in

syringyl content of the transgenic lignin (Figure 5). The lignin of the
F5H-overexpressing plants demonstrated a syringyl content as high as 29 mol% as
opposed to the syringyl content of the wild type lignin which was 18 mol%
(Table 1) (Example 5). These data clearly demonstrate that overexpression of the
5   F5H gene is useful for the alteration of lignin composition in transgenic plants.

TABLE 1
Impact of 35S Promoter-Driven F5H Expression on
Lignin Monomer Composition in *Arabidopsis*

Line	Total G units[a] (μmol g^{-1} d.w.)	Total S units[b] (μmol g^{-1} d.w.)	Total G+S units (μmol g^{-1} d.w.)	mol % S
wild type	3.33 +/- 0.32	0.75 +/- 0.09	4.09 +/- 0.41	18.4 +/- 0.91
fah1-2	5.44 +/- 0.45	n.d.	5.44 +/- 0.45	-
88	6.63 +/- 0.75	0.35 +/- 0.04	6.99 +/- 0.79	5.06 +/- 0.17
172	4.21 +/- 0.36	0.67 +/- 0.07	4.88 +/- 0.42	13.7 +/- 0.55
170	4.08 +/- 0.33	0.97 +/- 0.06	5.05 +/- 0.37	19.2 +/- 0.56
122	3.74 +/- 0.20	0.93 +/- 0.05	4.66 +/- 0.22	19.9 +/- 0.86
108	5.40 +/- 0.48	1.59 +/- 0.18	6.98 +/- 0.65	22.7 +/- 0.82
107	5.74 +/- 0.60	1.96 +/- 0.31	7.70 +/- 0.89	25.3 +/- 1.23
180	3.85 +/- 0.31	1.34 +/- 0.11	5.19 +/- 0.40	25.8 +/- 0.78
117	3.21 +/- 0.30	1.18 +/- 0.13	4.39 +/- 0.43	28.8 +/- 0.92
128	3.46 +/- 0.22	1.39 +/- 0.17	5.05 +/- 0.37	27.5 +/- 1.80

[a]sum of vanillin + vanillic acid

[b]sum of syringaldehyde + syringic acid

In a similar fashion, T1 tobacco (*Nicotiana tabacum*) F5H transformants
were generated, grown up and analyzed for lignin monomer composition.
10 Nitrobenzene oxidation analysis demonstrated that the syringyl monomer content
of the leaf midribs was increased from 14 mol% in the wild type to 40 mol% in the
transgenic line that most highly expressed the F5H transgene (Table 2).

TABLE 2
Impact of 35S Promoter-Driven F5H Expression on Lignin Monomer
Composition in Tobacco Leaf Midrib Xylem

Line	Total G units[a] (μmol g^{-1} d.w.)	Total S units[b] (μmol g^{-1} d.w.)	Total G+S units (μmol g^{-1} d.w.)	mol % S
wild type	1.40 +/- 0.26	0.23 +/- 0.04	1.63 +/- 0.30	14.3 +/- 1.09
40	0.86 +/- 0.16	0.24 +/- 0.03	1.11 +/- 0.20	22.4 +/- 1.53
27	1.13 +/- 0.11	0.52 +/- 0.05	1.65 +/- 0.16	31.3 +/- 0.50
48	1.28 +/- 0.32	0.71 +/- 0.19	1.99 +/- 0.43	35.7 +/- 6.06
33	0.65 +/- 0.17	0.43 +/- 0.11	1.09 +/- 0.27	40.0 +/- 1.86

[a]sum of vanillin + vanillic acid

[b]sum of syringaldehyde + syringic acid

Construction of Chimeric Genes for the Expression of F5H in Plants.

The expression of foreign genes in plants is well-established (De Blaere et
al. (1987) *Meth. Enzymol.* 143:277-291) and this invention provides for a method
5   to apply this technology to the introduction of a chimeric gene for the
overexpression of the F5H gene in plants for the manipulation of lignin monomer
composition. The expression of the F5H mRNAs at an appropriate level may
require the use of different chimeric genes utilizing different promoters. A
preferred class of heterologous hosts for the expression of the coding sequence of
10   the F5H gene are eukaryotic hosts, particularly the cells of higher plants.
Particularly preferred among the higher plants and the seeds derived from them are
alfalfa (*Medicago* sp.), rice (*Oryza* sp.), maize (*Zea mays*), oil seed rape (*Brassica*
sp.), forage grasses, and also tree crops such as eucalyptus (*Eucalyptus* sp.), pine
(*Pinus* sp.), spruce (*Picea* sp.) and poplar (*Populus* sp.), as well as *Arabidopsis* sp.
15   and tobacco (*Nicotiana* sp.). Expression in plants will use regulatory sequences
functional in such plants.

The origin of the promoter chosen to drive the expression of the coding
sequence is not critical as long as it has sufficient transcriptional activity to
accomplish the invention by expressing translatable mRNA for the F5H gene in the
20   desired host tissue. Preferred promoters will effectively target F5H expression to
those tissues that undergo lignification. These promoters may include, but are not
limited to promoters of genes encoding enzymes of the phenylpropanoid pathway
such as the PAL promoter (Ohl et al., *Plant Cell*, 2, 837, (1990) and the 4CL
promoter(Hauffed et al., *Plant Cell*, 3, 435, (1991).

25   Depending upon the application, it may be desirable to select promoters
that are specific for expression in one or more organs of the plant. Examples
include the light-inducible promoters of the small subunit of ribulose
1,5-bisphosphate carboxylase, if the expression is desired in photosynthetic organs,
or promoters active specifically in roots.

30   Expression of F5H Chimeric Genes in Plants

Various methods of introducing a DNA sequence (i.e., of transforming)
into eukaryotic cells of higher plants are available to those skilled in the art (see
EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include those
based on transformation vectors based on the Ti and Ri plasmids of *Agrobacterium*
35   *spp.* It is particularly preferred to use the binary type of these vectors. Ti-derived
vectors transform a wide variety of higher plants, including monocotyledonous and
dicotyledonous plants, such as soybean, cotton, tobacco, Arabidopsis and rape
(Pacciotti et al., *Bio/Technology* 3, 241, (1985); Byrne et al., *Plant Cell, Tissue
and Organ Culture* 8, 3, (1987); Sukhapinda et al., *Plant Mol. Biol.* 8, 209,

15

(1987); Lorz et al., *Mol. Gen. Genet.* 199, 178, (1985); Potrykus *Mol. Gen. Genet.* 199, 183, (1985)).

For introduction into plants the chimeric genes of the invention can be inserted into binary vectors as described in Example 5.

5 Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs [see EPO publication 0 295 959 A2], techniques of electroporation [see Fromm et al. (1986) *Nature* (London) 319:791] or high-velocity ballistic bombardment with metal particles coated with the nucleic acid constructs (see Kline et al., *Nature* (London) 327:70 (1987), and see U.S.

10 Patent No. 4,945,050). Once transformed, the cells can be regenerated by those skilled in the art.

The following Examples are meant to illustrate key embodiments of the invention but should not be construed to be limiting in any way.

EXAMPLES

15 GENERAL METHODS

Restriction enzyme digestions, phosphorylations, ligations and transformations were done as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press. All reagents and materials used for the growth and maintenance of bacterial cells

20 were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

The meaning of abbreviations is as follows: "h" means hour(s), "min" means minute(s), "sec" means second(s), "d" means day(s), "µL" means

25 microliter(s), "mL" means milliliters, "L" means liters, "g" means grams, "mg" means milligrams, "µg" means microgram(s), "nm" means nanometer(s), "m" means meter(s), "E" means Einstein(s).

Plant material

Arabidopsis thaliana was grown under a 16 h light/8 h dark photoperiod at

30 100 mE m^{-2} s^{-1} at 24 °C cultivated in Metromix 2000 potting mixture (Scotts, Marysville OH). Mutant lines *fahl-1* through *fahl-5* were identified by TLC as described below. Using their red fluorescence under UV light as a marker, mutant lines *fahl-6*, *fahl-7*, and *fahl-8* were selected from ethylmethane sulfonate (*fahl-6*, *fahl-7*) or fast-neutron (*fahl-8*) mutagenized populations of Landsberg *erecta* M2

35 seed. The T-DNA tagged line 3590 (*fahl-9*) was similarly identified in the DuPont T-DNA tagged population (Feldmann, K.A., Malmberg, R.L., & Dean, C., (1994) *Mutagenesis in Arabidopsis* in *Arabidopsis*, (E. M. Meyerowitz and C. R. Somerville, eds.) Cold Spring Harbor Press). All lines were backcrossed to wild

type at least twice prior to experimental use to remove unlinked background mutations.

Secondary Metabolite Analysis

Leaf extracts were prepared from 100 mg samples of fresh leaf tissue
5   suspended in 1 mL of 50% methanol. Samples were vortexed briefly, then frozen at -70 °C. Samples were thawed, vortexed, and centrifuged at 12,000 xg for 5 min. Sinapoylmalate content was qualitatively determined following silica gel TLC, in a mobile phase of n-butanol/ethanol/water (4:1:1). Sinapic acid and its esters were visualized under long wave UV light (365 nm) by their characteristic
10   fluorescence.

Southern Analysis

For Southern analysis, DNA was extracted from leaf material (Rogers, et al., (1985) *Plant. Mol. Biol. 5*, 69), digested with restriction endonucleases and transferred to Hybond N+ membrane (Amersham, Cleveland Ohio) by standard
15   protocols. cDNA probes were radiolabelled with ^{32}P and hybridized to the target membrane in Denhardt's hybridization buffer (900 mM sodium chloride, 6 mM disodium EDTA, 60 mM sodium phosphate pH 7.4, 0.5% SDS, 0.01% denatured herring sperm DNA and 0.1% each polyvinylpyrrolidone, bovine serum albumin, and Ficoll 400) containing 50% formamide at 42 °C. To remove unbound probe,
20   membranes were washed twice at room temperature and twice at 65 °C in 2x SSPE (300 mM sodium chloride, 2 mM disodium EDTA, 20 mM sodium phosphate, pH 7.4) containing 0.1% SDS, and exposed to film.

Northern Analysis

RNA was first extracted from leaf material according to the following
25   protocol.

For extraction of RNA, Covey's extraction buffer was prepared by dissolving 1% (w/v) TIPS (triisopropyl-naphthalene sulfonate, sodium salt), 6% (w/v) PAS (*p*-aminosalicylate, sodium salt) in 50 mM Tris pH 8.4 containing 5% v/v Kirby's phenol. Kirby's phenol was prepared by neutralizing liquified phenol
30   containing 0.1% (w/v) 8-hydroxyquinoline with 0.1 M Tris-HCl pH 8.8. For each RNA preparation, a 1 g samples of plant tissue was ground in liquid nitrogen and extracted in 5 mL Covey's extraction buffer containing 10 μL β-mercaptoethanol. The sample was extracted with 5 mL of a 1:1 mixture of Kirby's phenol and chloroform, vortexed, and centrifuged for 20 min at 7,000 xg. The supernatant
35   was removed and the nucleic acids were precipitated with 500 μL of 3 M sodium acetate and 5 mL isopropanol and collected by centrifugation at 10,000 xg for 10 min. The pellet was redissolved in 500 μL water, and the RNA was precipitated on ice with 250 μL 8 M LiCl, and collected by centrifugation at

10,000 xg for 10 min. The pellet was resuspended in 200 μL water and extracted with an equal volume of chloroform:isoamyl alcohol 1:1 with vortexing. After centrifugation for 2 min at 10,000 xg, the upper aqueous phase was removed, and the nucleic acids were precipitated at -20 °C by the addition of 20 μL 3 M sodium
5   acetate and 200 μL isopropanol. The pellet was washed with 1 mL cold 70% ethanol, dried, and resuspended in 100 μL water. RNA content was assayed spectrophotometrically at 260 nm. Samples containing 1 to 10 μg of RNA were subjected to denaturing gel electrophoresis as described elsewhere (Sambrook et al., *supra*).

10   Extracted RNA was transferred to Hybond N^+ membrane (Amersham, Cleveland Ohio), and probed with radiolabelled probes prepared from cDNA clones. Blots were hybridized overnight, washed twice at room temperature and once at 65 °C in 3x SSC (450 mM sodium chloride, 45 mM sodium citrate, pH 7.0) containing 0.1% SDS, and exposed to film.

15   Identification of cDNA and Genomic Clones

cDNA and genomic clones for F5H were identified by standard techniques using a 2.3 kb *SacII/EcoRI* fragment from the rescued plasmid (pCC1) (Example 2) as a probe. The cDNA clone pCC30 was identified in the λPRL2 library (Newman et al., (1994) supra) kindly provided by Dr. Thomas Newman
20   (DOE Plant Research Laboratory, Michigan State University, East Lansing, MI). A genomic cosmid library of *Arabidopsis thaliana* (ecotype Landsberg *erecta*) generated in the binary cosmid vector pBIC20 (Example 3) (Meyer et al., *Science* 264, 1452, (1994)) was screened with the radiolabelled cDNA insert derived from pCC30. Genomic inserts in the pBIC20 T-DNA are flanked by the neomycin
25   phosphotransferase gene for kanamycin selection adjacent to the T-DNA right border sequence, and the β-glucuronidase gene for histochemical selection adjacent to the left border. Positive clones were characterized by restriction digestion and Southern analysis in comparison to Arabidopsis genomic DNA.

Plant transformation

30   Transformation of *Arabidopsis thaliana* was performed by vacuum infiltration (Bent et al., *Science* 265, 1856, (1994)) with minor modifications. Briefly, 500 mL cultures of transformed *Agrobacterium* harboring the pBIC20-F5H cosmid or the pGA482-35S-F5H construct were grown to stationary phase in Luria broth containing 10 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin.
35   Cells were harvested by centrifugation and resuspended in 1 L infiltration media containing 2.2 g MS salts (Murashige and Skoog, *Physiol. Plant.* 15, 473, (1962)), Gamborg's B5 vitamins (Gamborg et al., *Exp. Cell Res.* 50, 151, (1968)), 0.5 g MES, 50 g sucrose, 44 nM benzylaminopurine, and 200 μL Silwet L-77 (OSI

Specialties) at pH 5.7. Bolting *Arabidopsis* plants (T_0 generation) that were 5 to
10 cm tall were inverted into the bacterial suspension and exposed to a vacuum
(>500 mm of Hg) for three to five min. Infiltrated plants were returned to standard
growth conditions for seed production. Transformed seedlings (T_1) were

5   identified by selection on MS medium containing 50 mg L^{-1} kanamycin and
200 mg L^{-1} timentin (SmithKline Beecham) and were transferred to soil.
        Transformation of tobacco was accomplished using the leaf disk method of
Horsch et al. (*Science* 227, 1229, (1985)).

Nitrobenzene oxidation

10   For the determination of lignin monomer composition, stem tissue was
ground to a powder in liquid nitrogen and extracted with 20 mL of 0.1 M sodium
phosphate buffer, pH 7.2 at 37 °C for 30 min followed by three extractions with
80% ethanol at 80 °C. The tissue was then extracted once with acetone and
completely dried. Tissue was saponified by treatment with 1.0 M NaOH at 37 °C

15   for 24 hours, washed three times with water, once with 80% ethanol, once with
acetone, and dried. Nitrobenzene oxidation of stem tissue samples was performed
with a protocol modified from Iiyama et al. (*J. Sci. Food Agric.* 51, 481-491.
(1990)). Samples of lignocellulosic material (5 mg each) were mixed with 500 μL
of 2 M NaOH and 25 μL of nitrobenzene. This mixture was incubated in a sealed

20   glass tube at 160 °C for 3 h. The reaction products were cooled to room
temperature and 5 μL of a 20 mg mL^{-1} solution of 3-ethoxy-4-hydroxybenzalde-
hyde in pyridine was added as an internal standard before the mixture was
extracted twice with 1 mL of dichloromethane. The aqueous phase was acidified
with HCl (pH 2) and extracted twice with 900 μL of ether. The combined ether

25   phases were dried with anhydrous sodium sulfate and the ether was evaporated in a
stream of nitrogen. The dried residue was resuspended in 50 μL of pyridine,
10 μL of BSA (N,O-bis-(trimethylsilyl)-trifluoracetamide) was added and 1 μL
aliquots of the silylated products were analyzed using a Hewlet-Packard 5890
Series II gas chromatograph equipped with Supelco SPB I column (30 m x

30   0.75 mm). Lignin monomer composition was calculated from the integrated areas
of the peaks representing the trimethylsilylated derivatives of vanillin,
syringaldehyde, vanillic acid and syringic acid. Total nitrobenzene oxidation-
susceptible guaiacyl units (vanillin and vanillic acid) and syringyl units
(syringaldehyde and syringic acid) were calculated following correction for

35   recovery efficiencies of each of the products during the extraction procedure
relative to the internal standard.

EXAMPLE 1

IDENTIFICATION OF THE T-DNA TAGGED ALLELE OF FAH1

A putatively T-DNA tagged *fah1* mutant was identified in a collection of T-DNA tagged lines (Feldmann et al., *Mol. Gen. Genet.* 208, 1, (1987)) (Dr. Tim

5   Caspar, Dupont, Wilmington, DE) by screening adult plants under long wave UV light. A red fluorescent line (line 3590) was selected, and its progeny were assayed for sinapoylmalate content by TLC. The analyses indicated that line 3590 did not accumulate sinapoylmalate. Reciprocal crosses of line 3590 to a *fah1-2* homozygote, followed by analysis of the F1 generation for sinapoylmalate content

10   demonstrated that line 3590 was a new allele of *fah1*, and it was designated *fah1-9*.

Preliminary experiments indicated co-segregation of the kanamycin-resistant phenotype of the T-DNA tagged mutant with the *fah1* phenotype. Selfed seed from 7 kanamycin-resistant [*fah1-9* x *FAH1*] F1 plants segregated 1:3 for kanamycin resistance ($kan^{sensitive}$ $kan^{resistant}$) and 3:1 for sinapoylmalate

15   deficiency (*FAH1:fah1*). From these lines, *fah1* plants gave rise to only $kan^{resistant}$, *fah1* progeny. To determine the genetic distance between the T-DNA insertion and the FAH1 locus, multiple test crosses were performed between a [*fah1-9* x *FAH1*] F1 and a *fah1-2* homozygote. The distance between the FAH1 locus and the T-DNA insertion was evaluated by determining the frequency at which

20     $FAH1/kan^{sensitive}$ progeny were recovered in the test cross F1. In the absence of crossover events, all kanamycin-resistant F1 progeny would be unable to accumulate sinapoylmalate, and would thus fluoresce red under UV light. In 682 $kan^{resistant}$ F1 progeny examined, no sinapoylmalate proficient plants were identified, indicating a very tight linkage between the T-DNA insertion site and the

25   FAH1 locus.

EXAMPLE 2

PLASMID RESCUE AND cDNA CLONING OF THE *fah1* GENE

Plasmid rescue was conducted using *EcoR*I-digested DNA prepared from homozygous *fah1-9* plants (Behringer et al., (1992), *supra*). Five μg of

30   *EcoR*I-digested genomic DNA was incubated with 125 U T4 DNA ligase overnight at 14 °C in a final volume of 1 mL. The ligation mixture was concentrated approximately four fold by two extractions with equal volumes of 2-butanol, and was then ethanol precipitated and electroporated into competent DH5-α cells as described (Newman et al., (1994), *supra*).

35   DNA from rescued plasmids was double digested with *EcoR*I and *Sal*I. Plasmids generated from internal T-DNA sequences were identified by the presence of triplet bands at 3.8, 2.4 and 1.2 kb and were discarded. One plasmid (pCC1) giving rise to the expected 3.8 kb band plus a novel 5.6 kb band was

identified as putative external right border plasmid. Using a *SacII/EcoRI* fragment of pCC1 that appeared to represent *Arabidopsis* DNA, putative cDNA (pCC30) clones for F5H were identified. The putative F5H clone carried a 1.9 kb *SalI-NotI* insert, the sequence of which was determined. Blastx analysis (Altschul et al., *J.*

5   *Mol. Biol.* 215, 403, (1990)) indicated that this cDNA encodes a cytochrome P450-dependent monooxygenase, consistent with earlier reports that (i) the *fah1* mutant is defective in F5H (Chapple et al., *supra*) and (ii) F5H is a cytochrome P450-dependent monooxygenase (Grand, *supra*).

Southern and Northern Blot analysis

10   To determine whether the putative F5H cDNA actually represented the gene that was disrupted in the T-DNA tagged line Southern and northern analysis was used to characterize the available *fah1* mutants using the putative F5H cDNA.

Figure 6 shows a Southern blot comparing hybridization of the F5H cDNA to *EcoRI*-digested genomic DNA isolated from wild type (ecotypes Columbia

15   (Col), Landsberg *erecta* (LER), and Wassilewskija (WS)) and the nine *fah1* alleles including the T-DNA tagged *fah1-9* allele. WS is the ecotype from which the T-DNA tagged line was generated.

These data indicated the presence of a restriction fragment length polymorphism between the tagged line and the wild type. These data also indicates

20   a restriction fragment length polymorphism in the *fah1-8* allele which was generated with fast neutrons, a technique reported to cause deletion mutations.

As shown in Figure 6 the genomic DNA of the *fah1-8* and *fah1-9* (the T-DNA tagged line) alleles is disrupted in the region corresponding to the putative F5H cDNA. These data also indicate that F5H is encoded by a single gene in

25   *Arabidopsis* as expected considering that the mutation in the *fah1* mutant segregates as a single Mendelian gene. These data provide the first indication that the putative F5H cDNA corresponds to the gene that is disrupted in the *fah1* mutants.

Plant material homozygous for nine independently-derived *fah1* alleles was

30   surveyed for the abundance of transcript corresponding to the putative F5H cDNA using Northern blot analysis. The data is shown in Figure 7.

As can be seen from the data, the putative F5H mRNA was represented at similar levels in leaf tissue of Columbia, Landsberg *erecta* and Wassilewskija ecotypes, and in the EMS-induced *fah1-1*, *fah1-4*, and *fah1-5*, as well as the fast

35   neutron-induced *fah1-7*. Transcript abundance was substantially reduced in leaves from plants homozygous for the *fah1-2*, *fah1-3* and *fah1-6*, all of which were EMS-induced, the fast neutron-induced mutant *fah1-8* and in the tagged line *fah1-9*. The mRNA in *fah1-8* mutant also appears to be truncated. These data provided

strong evidence that the cDNA clone that had been identified is encoded by the FAH1 locus.

EXAMPLE 3

DEMONSTRATION OF THE IDENTITY OF THE F5H cDNA BY
5   TRANSFORMATION OF *fah1* MUTANT PLANTS WITH WILDTYPE F5H
AND RESTORATION OF SINAPOYLMALATE ACCUMULATION

In order to demonstrate the identity of the F5H gene at the functional level, the transformation-competent pBIC20 cosmid library (Meyer et al., *supra*) was screened for corresponding genomic clones using the full length F5H cDNA as a

10   probe. A clone (pBIC20-F5H) carrying a genomic insert of 17 kb that contains 2.2 kb of sequence upstream of the putative F5H start codon and 12.5 kb of sequence downstream of the stop codon of the F5H gene (Figure 2) was transformed into the *fah1-2* mutant by vacuum infiltration. Thirty independent infiltration experiments were performed, and 167 kanamycin-resistant seedlings,

15   representing at least 3 transformants from each infiltration, were transferred to soil and were analyzed with respect to sinapic acid-derived secondary metabolites. Of these plants, 164 accumulated sinapoylmalate in their leaf tissue as determined by TLC (Figure 3). These complementation data indicate that the gene defective in the *fah1* mutant is present on the binary cosmid pBIC20-FSH.

20   To delimit the region of DNA on the pBIC20-FSH cosmid responsible for complementation of the mutant phenotype, a 2.7 kB fragment of the F5H genomic sequence was fused downstream of the cauliflower mosaic virus 35S promoter in the binary plasmid pGA482 and this construct (pGA482-35S-F5H) (Figure 2) was transformed into the *fah1-2* mutant. The presence of sinapoylmalate in 109 out of

25   110 transgenic lines analyzed by TLC or by in vivo fluorescence under UV light indicated that the *fah1* mutant phenotype had been complemented (Figure 3). These data provide conclusive evidence that the F5H cDNA has been identified.

EXAMPLE 4

DNA SEQUENCING OF THE F5H cDNA AND GENOMIC CLONES

30   The F5H cDNA and a 5156 bp *Hind*III-*Xho*I fragment of the pBIC20-F5H genomic clone were both fully sequenced on both strands and the sequence of the F5H protein (SEQ ID NO.:2) was inferred from the cDNA sequence (Figure 8). The sequence of the *Arabidopsis thaliana* F5H cDNA is given in SEQ ID NO.:1. The sequence of the *Arabidopsis thaliana* F5H genomic clone is given in SEQ ID

35   NO.:3.

EXAMPLE 5

MODIFICATION OF LIGNIN MONOMER COMPOSITION IN
TRANSGENIC PLANTS OVEREXPRESSING F5H

Generation of Transgenic Plants Ectopically Expressing the F5H Gene

5   Using an adaptor-based cloning strategy, regulatory sequences 5' of the
translation initiation site of the F5H gene were replaced with the strong
constitutive cauliflower mosaic virus 35S promoter (Odell et al., *Nature* 313,
810-812. (1985)), as shown in Figure 2. The resulting construct carries 2719 bp of
the F5H genomic sequence driven by the cauliflower mosaic virus 35S promoter
10   fused 50 bp upstream of the inferred ATG start codon. As a result, the cauliflower
mosaic virus 35S promoter drives the expression of the F5H gene by using the
transcription start site of the viral promoter and the termination signal present on
the F5H genomic sequence. This expression cassette for ectopic expression of
F5H was inserted into the T-DNA of the binary vector pGA482 (An, G. (1987),
15   *Binary Ti vectors for plant transformation and promoter analysis* in: Methods in
enzymology. Wu, R. ed. Academic Press, NY 153: 292-305) and introduced into
Agrobacterium tumefaciens by electroporation.

          Transgenic *Arabidopsis* plants of the ecotype Columbia that were
homozygous for the *fah1*-2 (Chapple et al., *supra*) allele were transformed with
20   *Agrobacterium* cultures harboring the pGA482-35S-F5H construct according to
the method of Bent et al. (*supra*). Transgenic plants of the T2 and T3 generation
were identified by selection on media containing kanamycin and subsequently
transferred to soil.

Determination of lignin monomer composition of *Arabidopsis* stem tissue

25   Total stem tissue was harvested from 4 week old plants that had been
grown in soil at 22 °C under a 16 h/8 h light/dark photoperiod. Nitrobenzene
oxidation analysis generated mol% syringyl values for 9 different transformant lines
(Table 1) ranging from 5.06 +/- 0.17 mol% to 28.8 +/- 0.92 mol% as opposed to
the wildtype control which demonstrated a value of 18.4 +/- 0.91 mol%. The
30   *fah1*-2 mutant background in which the transgenic lines were generated completely
lacks syringyl lignin (Table 1). The low expression of the F5H transgene in a
genetic background that lacks endogenous F5H message explains how line 88 can
have syringyl lignin levels that are lower than wild type.

          In addition to *Arabidopsis*, tobacco plants were transformed in a similar
35   fashion with the F5H gene under control of the cauliflower mosaic virus 35S
promoter. T2 and T3 positive transformants were screened and analyzed for lignin
modification and the data is given in Table 2. Nitrobenzene oxidation analysis of
tobacco leaf midribs generated mol% syringyl values for 4 different transformant

lines (Table 2) ranging from 22.4 +/- 1.53 mol% to 40.0 +/- 1.86 mol% as opposed
to the wildtype control which demonstrated a value of 14.3 +/- 1.09 mol%.

The data in Tables 1 and 2 clearly demonstrate that over-e xpression of the
F5H gene in transgenic plants results in the modification of lignin monomer
5   composition. The transformed plant is reasonably expected to have syringyl lignin
monomer content that is from about 0 mol% to about 95 mol% as measured in
whole plant tissue.

<u>SEQUENCE LISTING</u>

(1)   GENERAL INFORMATION:

    (i)   APPLICANT:
       (A)   NAME: PURDUE RESEARCH FOUNDATION
       (B)   STREET: 1650 ENGINEERING ADM BLDG, ROOM 328
       (C)   CITY: WEST LAFAYETTE
       (D)   STATE: INDIANA
       (E)   COUNTRY: UNITED STATES OF AMERICA
       (F)   POSTAL CODE (ZIP): 47907-1650
       (G)   TELEPHONE: 317-494-2610
       (H)   TELEFAX: 317-496-1277
       (I)   TELEX:

    (ii)   TITLE OF INVENTION: A METHOD FOR REGULATION OF
                                PLANT LIGNIN COMPOSITION

    (iii) NUMBER OF SEQUENCES: 3

    (iv)   COMPUTER READABLE FORM:
       (A)   MEDIUM TYPE: DISKETTE, 3.50 INCH
       (B)   COMPUTER: IBM PC COMPATIBLE
       (C)   OPERATING SYSTEM: WINDOWS 3.1
       (D)   SOFTWARE: MICROSOFT WORD 2.0C

    (v)   CURRENT APPLICATION DATA:
       (A)   APPLICATION NUMBER:
       (B)   FILING DATE:
       (C)   CLASSIFICATION:

    (vi)   PRIOR APPLICATION DATA:
       (A)   APPLICATION NUMBER: 60/009,119
       (B)   FILING DATE: DECEMBER 22, 1995

    (vii) ATTORNEY/AGENT INFORMATION:
       (A)   NAME: THOMAS Q. HENRY
       (B)   REGISTRATION NO.: 28,309
       (C)   REFERENCE/DOCKET NUMBER: CR-9870

(2)   INFORMATION FOR SEQ ID NO:1:

      (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 1838 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

      (ii)   MOLECULE TYPE: DNA (cDNA)

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
AAAAAAAACA CTCAATATGG AGTCTTCTAT ATCACAAACA CTAAGCAAAC TATCAGATCC    60

CACGACGTCT CTTGTCATCG TTGTCTCTCT TTTCATCTTC ATCAGCTTCA TCACACGGCG   120

GCGAAGGCCT CCATATCCTC CCGGTCCACG AGGTTGGCCC ATCATAGGCA ACATGTTAAT   180

GATGGACCAA CTCACCCACC GTGGTTTAGC CAATTTAGCT AAAAAGTATG GCGGATTGTG   240

CCATCTCCGC ATGGGATTCC TCCATATGTA CGCTGTCTCA TCACCCGAGG TGGCTCGACA   300

AGTCCTTCAA GTCCAAGACA GCGTCTTCTC GAACCGGCCT GCAACTATAG CTATAAGCTA   360

TCTGACTTAC GACCGAGCGG ACATGGCTTT CGCTCACTAC GGACCGTTTT GGAGACAGAT   420

GAGAAAAGTG TGTGTCATGA AGGTGTTTAG CCGTAAAAGA GCTGAGTCAT GGGCTTCAGT   480

TCGTGATGAA GTGGACAAAA TGGTCCGGTC GGTCTCTTGT AACGTTGGTA AGCCTATAAA   540

CGTCGGGGAG CAAATTTTTG CACTGACCCG CAACATAACT TACCGGGCAG CGTTTGGGTC   600

AGCCTGCGAG AAGGGACAAG ACGAGTTCAT AAGAATCTTA CAAGAGTTCT CTAAGCTTTT   660

TGGAGCCTTC AACGTAGCGG ATTTCATACC ATATTTCGGG TGGATCGATC CGCAAGGGAT   720

AAACAAGCGG CTCGTGAAGG CCCGTAATGA TCTAGACGGA TTTATTGACG ATATTATCGA   780

TGAACATATG AAGAAGAAGG AGAATCAAAA CGCTGTGGAT GATGGGGATG TTGTCGATAC   840

CGATATGGTT GATGATCTTC TTGCTTTTTA CAGTGAAGAG GCCAAATTAG TCAGTGAGAC   900

AGCGGATCTT CAAAATTCCA TCAAACTTAC CCGTGACAAT ATCAAAGCAA TCATCATGGA   960

CGTTATGTTT GGAGGAACGG AAACGGTAGC GTCGGCGATA GAGTGGGCCT TAACGGAGTT  1020

ATTACGGAGC CCCGAGGATC TAAAACGGGT CCAACAAGAA CTCGCCGAAG TCGTTGGACT  1080

TGACAGACGA GTTGAAGAAT CCGACATCGA GAAGTTGACT TATCTCAAAT GCACACTCAA  1140

AGAAACCCTA AGGATGCACC CACCGATCCC TCTCCTCCTC CACGAAACCG CGGAGGACAC  1200

TAGTATCGAC GGTTTCTTCA TTCCCAAGAA ATCTCGTGTG ATGATCAACG CGTTTGCCAT  1260

AGGACGCGAC CCAACCTCTT GGACTGACCC GGACACGTTT AGACCATCGA GGTTTTTGGA  1320

ACCGGGCGTA CCGGATTTCA AAGGGAGCAA TTTCGAGTTT ATACCGTTCG GGTCGGGTCG  1380

TAGATCGTGC CCGGGTATGC AACTAGGGTT ATACGCGCTT GACTTAGCCG TGGCTCATAT  1440

ATTACATTGC TTCACGTGGA AATTACCTGA TGGGATGAAA CCAAGTGAGC TCGACATGAA  1500

TGATGTGTTT GGTCTCACGG CTCCTAAAGC CACGCGGCTT TTCGCCGTGC CAACCACGCG  1560
```

CCTCATCTGT GCTCTTTAAG TTTATGGTTC GAGTCACGTG GCAGGGGGTT TGGTATGGTG 1620

AAAACTGAAA AGTTTGAAGT TGCCCTCATC GAGGATTTGT GGATGTCATA TGTATGTATG 1680

TGTATACACG TGTGTTCTGA TGAAAACAGA TTTGGCTCTT TGTTTGCCCT TTTTTTTTTT 1740

TTCTTTAATG GGGATTTTCC TTGAATGAAA TGTAACAGTA AAAATAAGAT TTTTTTCAAT 1800

AAGTAATTTA GCATGTTGCA AAAAAAAAAA AAAAAAAA   1838

(2)   INFORMATION FOR SEQ ID NO:2:

        (i)   SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 520 amino acids
              (B) TYPE: amino acid
              (C) STRANDEDNESS: unknown
              (D) TOPOLOGY: unknown

       (ii)   MOLECULE TYPE: protein

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Glu Ser Ser Ile Ser Gln Thr Leu Ser Lys Leu Ser Asp Pro Thr
1   5   10   15

Thr Ser Leu Val Ile Val Val Ser Leu Phe Ile Phe Ile Ser Phe Ile
            20   25   30

Thr Arg Arg Arg Arg Pro Pro Tyr Pro Pro Gly Pro Arg Gly Trp Pro
        35   40   45

Ile Ile Gly Asn Met Leu Met Met Asp Gln Leu Thr His Arg Gly Leu
    50   55   60

Ala Asn Leu Ala Lys Lys Tyr Gly Gly Leu Cys His Leu Arg Met Gly
65   70   75   80

Phe Leu His Met Tyr Ala Val Ser Ser Pro Glu Val Ala Arg Gln Val
            85   90   95

Leu Gln Val Gln Asp Ser Val Phe Ser Asn Arg Pro Ala Thr Ile Ala
            100   105   110

Ile Ser Tyr Leu Thr Tyr Asp Arg Ala Asp Met Ala Phe Ala His Tyr
        115   120   125

Gly Pro Phe Trp Arg Gln Met Arg Lys Val Cys Val Met Lys Val Phe
    130   135   140

Ser Arg Lys Arg Ala Glu Ser Trp Ala Ser Val Arg Asp Glu Val Asp
145   150   155   160

Lys Met Val Arg Ser Val Ser Cys Asn Val Gly Lys Pro Ile Asn Val
            165   170   175

Gly Glu Gln Ile Phe Ala Leu Thr Arg Asn Ile Thr Tyr Arg Ala Ala
            180   185   190

Phe Gly Ser Ala Cys Glu Lys Gly Gln Asp Glu Phe Ile Arg Ile Leu
        195   200   205

Gln Glu Phe Ser Lys Leu Phe Gly Ala Phe Asn Val Ala Asp Phe Ile
    210   215   220

27

```
Pro Tyr Phe Gly Trp Ile Asp Pro Gln Gly Ile Asn Lys Arg Leu Val
225             230             235             240

Lys Ala Arg Asn Asp Leu Asp Gly Phe Ile Asp Asp Ile Ile Asp Glu
            245             250             255

His Met Lys Lys Lys Glu Asn Gln Asn Ala Val Asp Asp Gly Asp Val
            260             265             270

Val Asp Thr Asp Met Val Asp Asp Leu Leu Ala Phe Tyr Ser Glu Glu
            275             280             285

Ala Lys Leu Val Ser Glu Thr Ala Asp Leu Gln Asn Ser Ile Lys Leu
    290             295             300

Thr Arg Asp Asn Ile Lys Ala Ile Ile Met Asp Val Met Phe Gly Gly
305             310             315             320

Thr Glu Thr Val Ala Ser Ala Ile Glu Trp Ala Leu Thr Glu Leu Leu
            325             330             335

Arg Ser Pro Glu Asp Leu Lys Arg Val Gln Gln Glu Leu Ala Glu Val
            340             345             350

Val Gly Leu Asp Arg Arg Val Glu Glu Ser Asp Ile Glu Lys Leu Thr
            355             360             365

Tyr Leu Lys Cys Thr Leu Lys Glu Thr Leu Arg Met His Pro Pro Ile
    370             375             380

Pro Leu Leu Leu His Glu Thr Ala Glu Asp Thr Ser Ile Asp Gly Phe
385             390             395             400

Phe Ile Pro Lys Lys Ser Arg Val Met Ile Asn Ala Phe Ala Ile Gly
            405             410             415

Arg Asp Pro Thr Ser Trp Thr Asp Pro Asp Thr Phe Arg Pro Ser Arg
            420             425             430

Phe Leu Glu Pro Gly Val Pro Asp Phe Lys Gly Ser Asn Phe Glu Phe
    435             440             445

Ile Pro Phe Gly Ser Gly Arg Arg Ser Cys Pro Gly Met Gln Leu Gly
    450             455             460

Leu Tyr Ala Leu Asp Leu Ala Val Ala His Ile Leu His Cys Phe Thr
465             470             475             480

Trp Lys Leu Pro Asp Gly Met Lys Pro Ser Glu Leu Asp Met Asn Asp
            485             490             495

Val Phe Gly Leu Thr Ala Pro Lys Ala Thr Arg Leu Phe Ala Val Pro
            500             505             510

Thr Thr Arg Leu Ile Cys Ala Leu
        515             520
```

(2)   INFORMATION FOR SEQ ID NO:3:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 5156 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

(ii)   MOLECULE TYPE: DNA (genomic)

(xi)   SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AAGCTTATGT ATTTCCTTAT AACCATTTTA TTCTGTATAT AGGGGGACAG AAACATAATA    60
AGTAACAAAT AGTGGTTTTA TTTTTTTAAA TATACAAAAA CTGTTTAACC ATTTTATTTC   120
TTGGTTAGCA AAATTTTGAT ATATTCTTAA GAAACTAATA TTTTAGGTTG ATATATTGCA   180
GTCACTAAAT AGTTTTAAAA GACACGAAGT TGGTAAGAAC AGGCATATAT TATTCGATTT   240
AATTAGGAAT GCTTATGTTA ATCTGATTCG ACTAATTAGA AACGACGATA CTATGAGCTC   300
ATAGATGGTC CCACGACCCA CTCTCCCATT TGATCAATAT TCAACTGAGC AATGAAACTA   360
ATTAAAAACG TGGTTAGATT AAAAAAATAA ATTGTGCAGG TAGCGGATAT ATAATACTAG   420
TAGGGGTTAA AAATAAAATA AAACACCACA GTATTAAATT TTTGTTTCAA AAGTATTATC   480
AATAGTTTTT TTGCTTCAAA AATATCACAA ATTTTTGTAT GAAATATTTC TTTAACGAAA   540
ATAAATTAAA TAAAATTTAA AATTTATATT TGGAGTTCTA TTTTTAATTT AGAGTTTTTA   600
TTGTTACCAC ATTTTTTGAA TTATTCTAAT ATTAATTTGT GATATTATTA CAAAAAGTAA   660
AAATATGATA TTTTAGAATA CTATTATCGA TATTTGATAT TATTGACCTT AGCTTTGTTT   720
GGGTGGAGAC ATGTGATTAT CTTATTACCT TTTTATTCCA TGAAACTACA GAGTTCGCCA   780
GGTACCATAC ATGCACACAC CCTCGTGAAG CCGTGACTTA ATATGATCTA GAACTTAAAT   840
AGTACTACTA ATTGTGTCAT TTGAACTTTC TCCTATGTCG GTTTCACTTC ATGTATCGCA   900
GAACAGGTGG AATACAGTGT CCTTGAGTTT CACCCAAATC GGTCCAATTT TGTGATATAT   960
ATTGCGATAC AGACATACAG CCTACAGAGT TTTGTCTTAG CCCACTGGTT GGCAAACGAA  1020
ATTGTCTTTA TTTTTTTATG TTTTGTTGTC AATGTGTCTT TGTTTTTAAC TAGATTGAGG  1080
TTTAATTTTA ATACATTTGT TAGTTTACAG ATTATGCAGT GTAATCTGAT AATGTAAGTT  1140
GAACTGCGTT GGTCAAAGTC TTGTGTAACG CACTGTATCT AAATTGTGAG TAACGACAAA  1200
ATAATTAAAA TTAAAGGACC TTCAAGTATT ATTAGTATCT CTGTCTAAGA TGCACAGGTA  1260
TTCAGTAATA GTAATAAATA ATTACTTGTA TAATTAATAT CTAATTAGTA AACCTTGTGT  1320
CTAAACCTAA ATGAGCATAA ATCCAAAAGC AAAAATCTAA ACCTAACTGA AAAAGTCATT  1380
ACGAAAAAAA GAAAAAAAAA AGAGAAAAAA CTACCTGAAA AGTCATGCAC AACGTTCATC  1440
TTGGCTAAAT TTATTTAGTT TATTAAATAC AAAAATGGCG AGTTCTGGA GTTGTTGAA   1500
AATATATTTG TTTAGCCACT TTAGAATTTC TTGTTTTAAT TTGTTATTAA GATATATCGA  1560
GATAATGCGT TTATATCACC AATATTTTTG CCAAACTAGT CCTATACAGT CATTTTTCAA  1620
CAGCTATGTT CACTAATTTA AAACCCACTG AAAGTCAATC ATGATTCGTC ATATTTATAT  1680
GCTCGAATTC AGTAAAATCC GTTTGGTATA CTATTTATTT CGTATAAGTA TGTAATTCCA  1740
CTAGATTTCC TTAAACTAAA TTATATATTT ACATAATTGT TTTCTTTAAA AGTCTACAAC  1800
AGTTATTAAG TTATAGGAAA TTATTTCTTT TATTTTTTTT TTTTTTTAGG AAATTATTTC  1860
TTTTGCAACA CATTTGTCGT TTGCAAACTT TTAAAAGAAA ATAAATGATT GTTATAATTG  1920
ATTACATTTC AGTTTATGAC AGATTTTTTT TATCTAACCT TTAATGTTTG TTTCCCTGTT  1980
TTTAGGAAAA TCATACCAAA ATATATTTGT GATCACAGTA AATCACGGAA TAGTTATGAC  2040
CAAGATTTTC AAAGTAATAC TTAGAATCCT ATTAAATAAA CGAAATTTTA GGAAGAAATA  2100
```

```
ATCAAGATTT TAGGAAACGA TTTGAGCAAG GATTTAGAAG ATTTGAATCT TTAATTAAAT  2160
ATTTTCATTC CTAAATAATT AATGCTAGTG GCATAATATT GTAAATAAGT TCAAGTACAT  2220
GATTAATTTG TTAAAATGGT TGAAAAATAT ATATATGTAG ATTTTTTCAA AAGGTATACT  2280
AATTATTTTC ATATTTTCAA GAAAATATAA GAAATGGTGT GTACATATAT GGATGAAGAA  2340
ATTTAAGTAG ATAATACAAA AATGTCAAAA AAAGGGACCA CACAATTTGA TTATAAAACC  2400
TACCTCTCTA ATCACATCCC AAAATGGAGA ACTTTGCCTC CTGACAACAT TTCAGAAAAT  2460
AATCGAATCC AAAAAAAACA CTCAATATGG AGTCTTCTAT ATCACAAACA CTAAGCAAAC  2520
TATCAGATCC CACGACGTCT CTTGTCATCG TTGTCTCTCT TTTCATCTTC ATCAGCTTCA  2580
TCACACGGCG GCGAAGGCCT CCATATCCTC CCGGTCCACG AGGTTGGCCC ATCATAGGCA  2640
ACATGTTAAT GATGGACCAA CTCACCCACC GTGGTTTAGC CAATTTAGCT AAAAAGTATG  2700
GCGGATTGTG CCATCTCCGC ATGGGATTCC TCCATATGTA CGCTGTCTCA TCACCCGAGG  2760
TGGCTCGACA AGTCCTTCAA GTCCAAGACA GCGTCTTCTC GAACCGGCCT GCAACTATAG  2820
CTATAAGCTA TCTGACTTAC GACCGAGCGG ACATGGCTTT CGCTCACTAC GGACCGTTTT  2880
GGAGACAGAT GAGAAAGTG TGTGTCATGA AGGTGTTTAG CCGTAAAAGA GCTGAGTCAT  2940
GGGCTTCAGT TCGTGATGAA GTGGACAAAA TGGTCCGGTC GGTCTCTTGT AACGTTGGTA  3000
AGCTACTTCA CATATTCACC ACTCTTGCTA TATATATGTG CAATTAAACA AATATGTAAA  3060
AAGTGAAAGT ACTCATTTCT TCTTTCTTTA GTATGTACTT TAACATTTAA CCAAAACAAT  3120
TGTAGGTAAG CCTATAAACG TCGGGGAGCA AATTTTTGCA CTGACCCGCA ACATAACTTA  3180
CCGGGCAGCG TTTGGGTCAG CCTGCGAGAA GGGACAAGAC GAGTTCATAA GAATCTTACA  3240
AGAGTTCTCT AAGCTTTTTG GAGCCTTCAA CGTAGCGGAT TTCATACCAT ATTTCGGGTG  3300
GATCGATCCG CAAGGGATAA ACAAGCGGCT CGTGAAGGCC CGTAATGATC TAGACGGATT  3360
TATTGACGAT ATTATCGATG AACATATGAA GAAGAAGGAG AATCAAAACG CTGTGGATGA  3420
TGGGGATGTT GTCGATACCG ATATGGTTGA TGATCTTCTT GCTTTTTACA GTGAAGAGGC  3480
CAAATTAGTC AGTGAGACAG CGGATCTTCA AAATTCCATC AAACTTACCC GTGACAATAT  3540
CAAAGCAATC ATCATGGTAA TTATATTTCA AAAAGCACTA GTCATAGTCA TGTTTCTTAA  3600
TGCGTTACGT AATAATACTT ATCCATTGAC CAGTTATTTT CTCCTAAGTT TTTTTGTTTG  3660
AATTAGGAAG GTAATTTTCT ATTTTACTAG AGAAAGCAAC AGATTTTAGC ATGATCTTTT  3720
TTTAATATAT ATAGAAGCAT TGAATATTCA GATCTACAAT AATTATGAAA CTAATGAAGA  3780
GACAAAAAAT GGAGAGAGAA AAAAGAAAGA GTGGACTAGT GTGGATATAT TTAATTCTAA  3840
TTTGATTTTA TTAGGACGTT ATATTTAATT CTAATTTGAT TTTTTATTTT GATTTTATTA  3900
GGACGTTATG TTTGGAGGAA CGGAAACGGT AGCGTCGGCG ATAGAGTGGG CCTTAACGGA  3960
GTTATTACGG AGCCCCGAGG ATCTAAAACG GGTCCAACAA GAACTCGCCG AAGTCGTTGG  4020
ACTTGACAGA CGAGTTGAAG AATCCGACAT CGAGAAGTTG ACTTATCTCA AATGCACACT  4080
CAAAGAAACC CTAAGGATGC ACCCACCGAT CCCTCTCCTC CTCCACGAAA CCGCGGAGGA  4140
CACTAGTATC GACGGTTTCT TCATTCCCAA GAAATCTCGT GTGATGATCA ACGCGTTTGC  4200
CATAGGACGC GACCCAACCT CTTGGACTGA CCCGGACACG TTTAGACCAT CGAGGTTTTT  4260
GGAACCGGGC GTACCGGATT TCAAAGGGAG CAATTTCGAG TTTATACCGT TCGGGTCGGG  4320
TCGTAGATCG TGCCCGGGTA TGCAACTAGG GTTATACGCG CTTGACTTAG CCGTGGCTCA  4380
```

30

```
TATATTACAT TGCTTCACGT GGAAATTACC TGATGGGATG AAACCAAGTG AGCTCGACAT   4440
GAATGATGTG TTTGGTCTCA CGGCTCCTAA AGCCACGCGG CTTTTCGCCG TGCCAACCAC   4500
GCGCCTCATC TGTGCTCTTT AAGTTTATGG TTCGAGTCAC GTGGCAGGGG GTTTGGTATG   4560
GTGAAAACTG AAAAGTTTGA AGTTGCCCTC ATCGAGGATT TGTGGATGTC ATATGTATGT   4620
ATGTGTATAC ACGTGTGTTC TGATGAAAAC AGATTTGGCT CTTTGTTTGC CCTTTTTTTT   4680
TTTTTCTTTA ATGGGGATTT TCCTTGAATG AAATGTAACA GTAAAAATAA GATTTTTTTC   4740
AATAAGTAAT TTAGCATGTT GCAAAGATCG ATCTTGGATG AGAACTTCTA CTTAAAAAAA   4800
AAAAAAAAAT TTTTTTTTAG TTATTTCACC TTTTTCTTTT GTTCTGGTTG TATGGTTGCC   4860
ATTGTGTCAA TTAGGGGCTG GAAGTTCGCT GGTTAAGGCT AAATCAGAGT TAAAGTTATA   4920
ATTTTACAAG CCCAACAAAA GGTCGCAGAT TAAAACCACA TGATATTTAT AAAAAAAATT   4980
CTAAGGTTTT TATTAGTTTT ATTTTCAGTT TACTGAGTAC TATTTACTTT TTTATTTTTT   5040
GCAAATAAAT GTATTTATC ATATTTATGT TTTTTGTTAT AAACTCCAAA CATACAGGTT   5100
TCATTACCTA AAAAAAGACA GAGTGGTTTC GTTAATTTTG TTTCATTAAT CTCGAG       5156
```

WHAT IS CLAIMED IS:

1. An isolated nucleic-acid fragment encoding an active plant F5H enzyme having an amino acid sequence encoded by a mature functional protein which corresponds to SEQ ID NO.:2 and wherein the amino acid sequence encompasses amino acid substitutions, additions and deletions that do not alter the function of the active plant F5H enzyme.
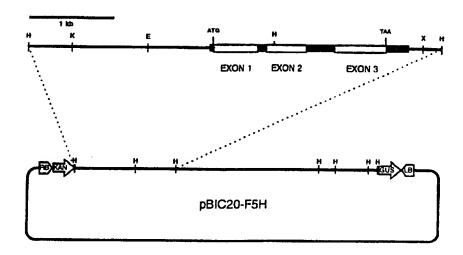
2. An isolated nucleic-acid fragment selected from the group consisting of nucleic acid fragments corresponding to SEQ ID NO.:1 and SEQ ID NO.:3.

3. A chimeric gene causing altered guaiacyl:syringyl lignin monomer ratios in a plant cell transformed with the chimeric gene, the chimeric gene comprising the nucleic acid fragment of Claims 1 or 2 operably linked in either sense or antisense orientation to at least one suitable regulatory sequence.

4. The chimeric gene of Claim 3 wherein the nucleic acid fragment is operably linked in the sense orientation to at least one suitable regulatory sequence.

5. The chimeric gene of Claims 3 wherein the at least one regulatory sequence comprises a promoter selected from the group consisting of cauliflower mosaic virus 35S promoter, the promoter for the phenylalanine ammonia lyase gene, the promoter for the p-coumaroyl CoA ligase gene, and endogenous plant promoters capable of controlling expression of plant F5H genes.

6. A transformed plant having altered guaiacyl:syringyl lignin monomer ratios relative to the ratios of an untransformed plant and comprising a suitable host plant and the chimeric gene of Claims 3.

7. The transformed plant of Claim 6 wherein the syringyl lignin monomer content is from about 0 mol% to about 95 mol% as measured in whole plant tissue.

8. The transformed plant of Claim 7 wherein the suitable host plant is selected from the group consisting of alfalfa (Medicago sp.), rice (Oryza sp.), maize (Zea mays), oil seed rape (Brassica sp.), forage grasses, Arabidopsis sp., tobacco (Nicotiana sp.) and tree crops such as eucalyptus (Eucalyptus sp.), pine (Pinus sp.), spruce (Picea sp.) and poplar (Populus sp.).

9. A method of altering the activity of F5H enzyme in a plant, comprising:

(i)   transforming a cell, tissue or organ from a suitable host plant with the chimeric gene of Claim 3 wherein the chimeric gene is expressed;

(ii)   selecting transformed cells, cell callus, somatic embryos, or seeds which contain the chimeric gene;

(iii)   regenerating whole plants from the transformed cells, cell callus, somatic embryos, or seeds selected in step (ii);

(iv)   selecting whole plants regenerated in step (iii) which have a phenotype characterized by (1) an ability of the whole plant to accumulate

compounds derived from sinapic acid or (2) an altered syringyl lignin monomer content relative to an untransformed host plant.

10. A method of altering the content or composition of lignin in a plant, comprising stably incorporating the chimeric gene of Claim 3 into the genome of the host plant by transformation means whereby the incorporated chimeric gene expresses F5H enzyme and whereby guaiacyl:syringyl lignin monomer ratios are altered from those of the untransformed host plant.
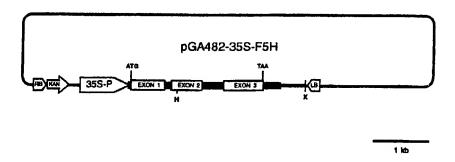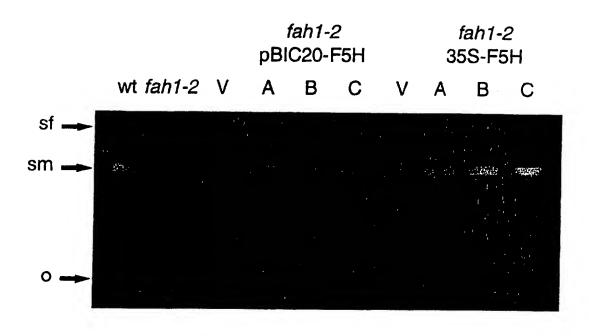
Figure 1

1/8

Figure 2

2/8

Figure 3

24 hour exposure
1. wild type
2. *fah1-2*

2 hour exposure
3. wild type
4. *fah1-2*
5. line 88
6. line 172
7. line 170
8. line 122
9. line 128
10. line 107
11. line 180
12. line 117
13. line 108

Figure 4

relative
detector
response

Figure 5

5/8

Figure 6

6/8

Figure 7

7/8

ATG   TAA

| exon 1 | exon 2 | exon 3 |

H   K   H   X

Figure 8